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Review

Turning Over a New Leaf in Lipid Droplet Biology

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Lipid droplets (LDs) in plants have long been viewed as storage depots for neutral lipids that serve as sources of carbon, energy, and lipids for membrane biosynthesis. While much of our knowledge of LD function in plants comes from studies of oilseeds, a recent surge in research on LDs in non-seed cell types has led to an array of new discoveries. It is now clear that both evolutionarily conserved and kingdom-specific mechanisms underlie the biogenesis of LDs in eukaryotes, and proteomics and homology-based approaches have identified new protein players. This review highlights some of these recent discoveries and other new areas of plant LD research, including their role in stress responses and as targets of metabolic engineering strategies aimed at increasing oil content in bioenergy crops.

Trends

In addition to the traditional role of storing energy-rich neutral lipids in seeds, cytoplasmic lipid droplets (LDs) are involved in a remarkably wide variety of other cellular processes throughout the plant life cycle, including stress responses, hormone metabolism, organ development, and lipid signaling.

The biogenesis of LDs (i.e., their formation, maintenance, and turnover) in plant cells involves both evolutionarily conserved and organism/tissue-specific mechanisms.

Recent proteomic analyses of LDs, particularly those involving non-seed tissues, as well as searches for homologs of lipodystrophy-related proteins responsible for storage lipid disorders in humans have led to the discovery of several new protein components in plant LD biology.

LDs represent an attractive platform for bioengineering strategies aimed at elevating the accumulation of biofuels and lipophilic bioproducts in plants.

LDs in Seeds and Vegetative Organs of Plants

Neutral lipids (see Glossary) are involved in a myriad of cellular functions, including energy homeostasis, membrane remodeling, and lipid signaling. While small quantities of neutral lipids are often present in cellular membranes, mechanisms have evolved to package and store neutral lipids into discrete compartments called **LDs** (also referred to as lipid/oil bodies, oleosomes, and spherosomes). Found in all kingdoms of life, including prokaryotes and eukaryotes [1–7], LDs have a unique but relatively simple organizational structure comprising a core of non-bilayer lipids such as triacylglycerols (TAGs) and sterol esters (StEs) surrounded by a phospholipid monolayer and various surface-associated 'coat' proteins (Figure 1A) [8,9].

In plants LDs are formed in at least two subcellular locations, the chloroplast and the endoplasmic reticulum (ER). In chloroplasts LDs form by localized accumulation of neutral lipids between the membrane leaflets of the thylakoid cisternae and then pinch off into the stroma to form particles called plastoglobuli [10]. Similarly, in certain algae LDs may bud from the cytoplasmic surface of the chloroplast outer envelope membrane [11,12]. LDs also form within the bilayer of the ER membrane and pinch off into the cytoplasm, and while relatively little is known about the mechanistic details of this process in plants, recent studies with mammalian, yeast, and insect cells have begun to shed considerable light on the key steps and proteins involved in cytoplasmic LD formation in these organisms and how, conceptually, the process appears to be relatively well conserved (Box 1).

Much of our knowledge of ER-derived cytoplasmic LDs in plants comes from studies of pollen grains and oilseeds, the latter of which accumulate large numbers of LDs storing energy-dense TAGs needed to support post-germinative seedling growth (Figure 1B). The LDs of oilseeds are easily isolated [13] and investigations of their protein content have revealed that the most abundant protein constituents are the oleosins [14,15]. Oleosins are important for stabilizing

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LDs, particularly during seed (and pollen grain) desiccation [16–20], and have also been implicated in the formation of LDs at the ER [17] and LD turnover [21]. Oleosins are specific to plants [22], including green algae [23], but in mammals and insects there is a different set of abundant LD proteins (i.e., the perilipins) [24] (Box 1) that are absent from plants but serve similar functions. Thus, while the general process of neutral lipid compartmentalization is conserved functionally between organisms, the LD coat, as well as other aspects of **LD biogenesis**, may be performed by distinctly different sets of proteins.

ER-derived cytoplasmic LDs also are found in cells of the vegetative organs of plants [1,2], including those in roots, stems, and leaves (Figure 1B). However, since oleosins are much less abundant or even absent in these cell types, they are likely to possess other LD-associated proteins involved in the biogenesis and function of LDs. Recent proteomics and homology-based studies have led to the identification of several new protein components involved in the formation, maintenance, and/or turnover of LDs (Table 1). Analysis of these proteins has further illuminated new roles for LDs in plant biology, providing evidence that LDs are not simply static depots of energy-rich neutral lipids but rather *bona fide* organelles involved in a dynamic array of cellular and physiological processes such as biotic and abiotic stress responses.

This review summarizes these recent findings, identifies new and emerging areas of research in LD biogenesis and function, and highlights several biotechnology-oriented approaches to enhance the neutral lipid content and composition in plant vegetative organs and seeds through targeted engineering of the LD compartment itself.

Proteomics Reveals New Players in Plant LD Biology

While LDs have been studied largely in the context of lipid storage and turnover in oilseeds, virtually all plant cell types have the machinery required to synthesize and compartmentalize TAGs into cytoplasmic LDs. To help identify proteins involved in LD biogenesis and function in non-seed cell types, Horn *et al.* [25] conducted a proteomics analysis of LDs isolated from avocado mesocarp, the oil-rich non-seed portion of the avocado fruit that is almost completely devoid of oleosins. These studies revealed numerous proteins associated with avocado mesocarp LDs, and surprisingly two of the five most abundant protein candidates were

Glossary

Biofuels and bioproducts: carbon-based materials derived from biological sources (e.g., plants, algae) that substitute for similar compounds or products derived from fossil oil.
Lipid droplet (LD) biogenesis: all of the cellular events involved in the formation, maintenance, and turnover of LDs.
Lipid droplets (LDs): subcellular organelles that store a variety of neutral lipid compounds, such as DAGs, TAGs, StEs, polyisoprenes, and certain pigments (e.g., β-carotene); unique among organelles in that they are delineated by a phospholipid monolayer and form an emulsion in the cell's interior, allowing the accumulation of large amounts of neutral lipids while avoiding disruption to cellular membranes. LDs in plants are classically referred to as oil bodies, oil droplets, oleosomes, or spherosomes; referred to as rubber particles in rubber-producing plants and as plastoglobules in plastids.
Lipolysis: the regulated pathway involved in the metabolism (hydrolysis) of stored neutral lipids in LDs via cellular lipases.
Neutral lipids: molecules that are inherently hydrophobic in nature and typically present in minor amounts in cellular membranes. Common examples in plants include DAGs, TAGs, StEs, and polyisoprenes.

Box 1. LD Biogenesis in Mammals, Insects, and Yeast

Recent studies with mammalian, insect, and yeast cells have provided unprecedented insight on several discrete steps in the biogenesis of LDs (reviewed in [5–7,101–106]). Neutral lipids such as TAGs are first synthesized by ER-localized enzymes, including glycerol-3-phosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT), and then accumulate between the two leaflets of the ER membrane to form a lens-like structure (Figure I). An ER membrane protein, FIT2, then binds and delivers additional TAG to the 'lens', promoting its growth and vectorial budding from the membrane surface, which may occur at specialized sites (subdomains) of the ER. Lipins, which are phosphatidate phosphatases, are recruited for the synthesis of DAG, which serves as a substrate for TAG production and is incorporated into the phospholipid monolayer of the nascent LD. The unique shape of diacylglycerol (DAG) also promotes and maintains the curvature of the membrane and helps to recruit structural coat proteins such as perilipins.

Conversion of nascent LDs into mature LDs requires SEIPIN, which is an ER membrane protein that localizes specifically to and stabilizes ER–LD junction sites. SEIPIN functions in part by facilitating the incorporation of additional proteins and lipids into the growing LD. In doing so SEIPINs influence both the number and size of LDs and also help determine the phospholipid composition of the LD monolayer. CTP:phosphocholine cytidylyltransferase 1 (CCT1) is recruited to the LD surface to help coordinate the synthesis of phospholipids in the monolayer with that of the expanding neutral lipid core.

Mature LDs can eventually detach by some unknown process from the ER into the cytoplasm, where they can interact with various other organelles and/or grow by fusion, either through coalescence or the transfer of neutral lipids from smaller to larger LDs, a process mediated by FSP27. LDs can also be eventually degraded by cytoplasmic neutral lipid lipases (i.e., lipolysis) or autophagy (i.e., lipophagy) [59]. Alternatively, LDs can maintain a permanent physical connection with the ER throughout their life cycle, allowing them to rapidly grow and shrink in response to the needs of the cell/organism.

When cellular cues promote the synthesis of large amounts of neutral storage lipids (e.g., caloric excess), a subset of mature LDs can be converted to 'expanding' LDs. Here the mature LD reconnects with the ER and TAG biosynthetic enzymes (i.e., DGATs and GPATs) migrate from the ER to the surface of the LD to facilitate both localized neutral lipid synthesis and LD expansion. The formation of ER–LD connections is regulated by the Arf1–coatomer I (COPI) machinery, the transport protein particle II complex, and Rab proteins. Protein shuttling between the ER and expanding LDs is also mediated by Arf1–COPI as well as by distinct hydrophobic hairpin motifs. Other specific molecular targeting signals (e.g., amphipathic helices) mediate the localization of proteins that sort from the cytoplasm to LDs, such as perilipins and CCT1.

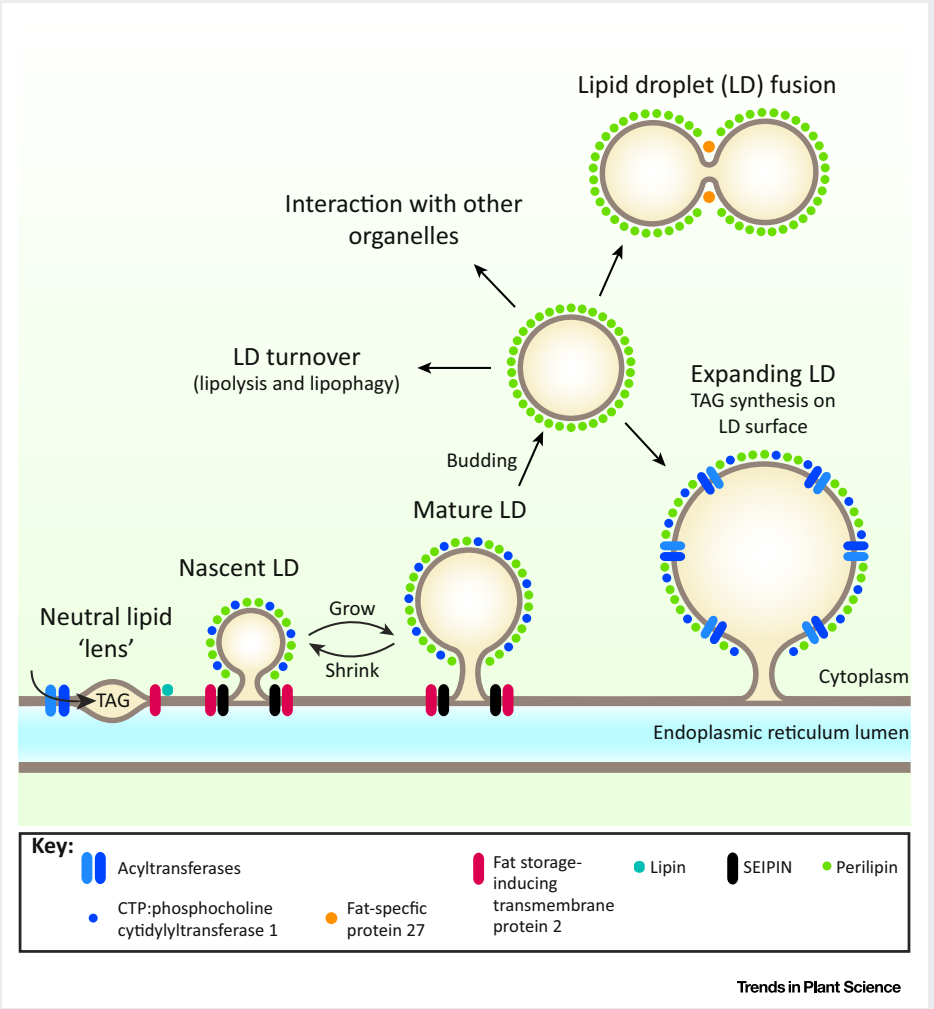


Figure I. Model of LD Biogenesis.

annotated as small rubber particle proteins (SRPPs) [25]. The SRPPs are involved in the formation and stabilization of polyisoprenoid-containing LDs in rubber-producing plants such as *Hevea brasiliensis* [26]. Given that avocado mesocarp lacks rubber, these observations suggested that the TAG-containing LDs in avocado and rubber-containing LDs in rubber-producing plants are functionally similar organelles, a premise subsequently supported by the reported localization of *H. brasiliensis* SRPPs to TAG-containing LDs in tobacco cultured cells, which lack rubber particles [27].

Further characterization of the *SRPP*-like genes in arabidopsis (*Arabidopsis thaliana*) revealed a three-member protein family that, because arabidopsis lacks rubber, were termed

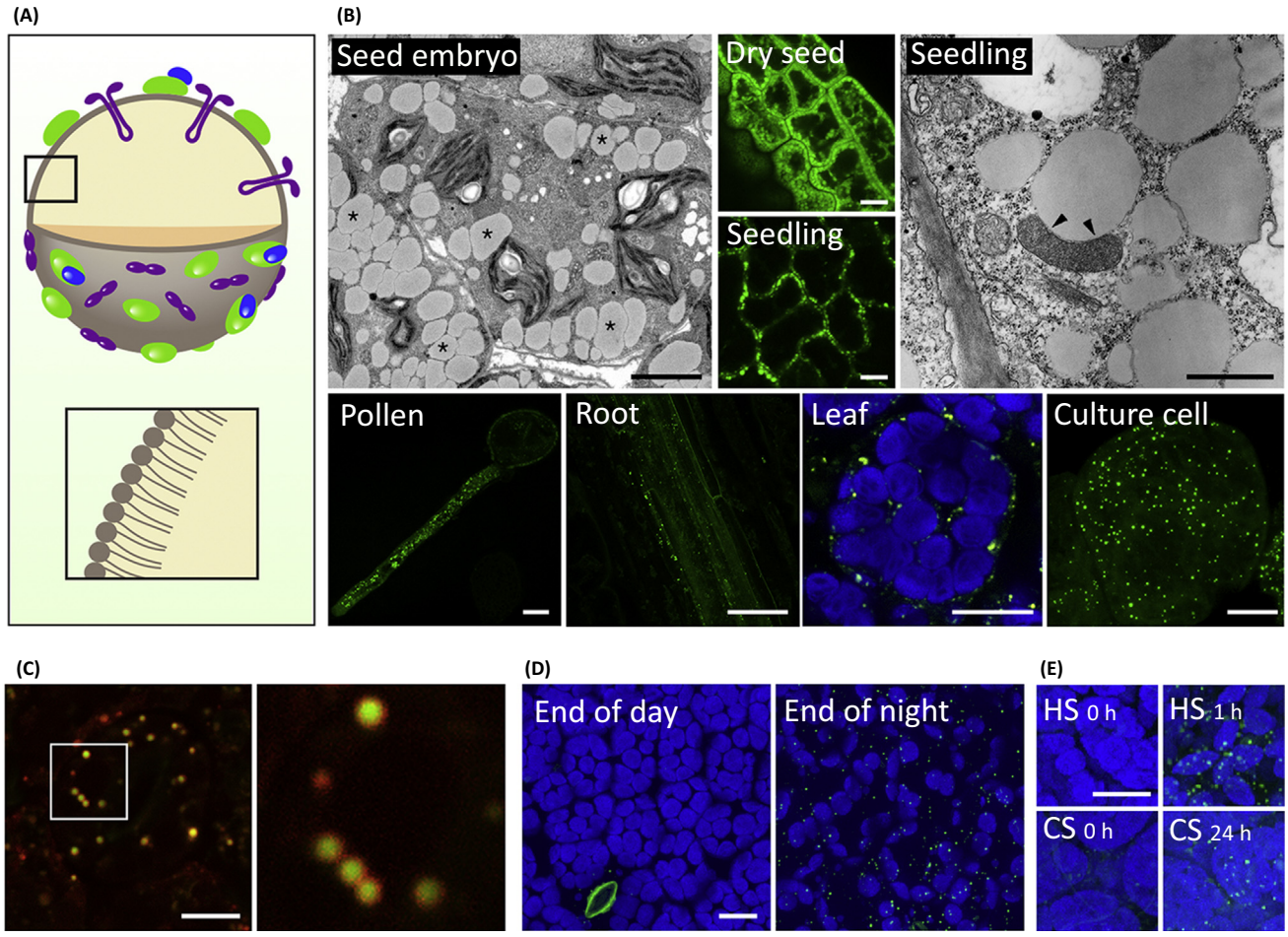


Figure 1. Plant Lipid Droplets (LDs). (A) Schematic representation of the structure of a LD. LDs possess a central core of neutral lipids [i.e., triacylglycerols (TAGs) and sterol esters (StEs)] surrounded by a phospholipid monolayer (insert). LD 'coat' proteins either integrate into the LD core via a hydrophobic hairpin domain (purple) or associate in a peripheral manner with the LD surface via their interaction with the charged phospholipid head groups (green) and/or other proteins (blue). (B) LDs in cells of seeds and various non-seed organs, tissues, or cells. Top row, left panel: Transmission electron microscopy (TEM) image of cotyledon cells in an arabidopsis (*Arabidopsis thaliana*) developing seed embryo, when the embryo is actively synthesizing storage oil. For reference purposes some LDs are indicated with asterisks. Bar, 2 μm. Top row, middle two panels: Confocal laser-scanning microscopy (CLSM) images (shown as Z-stack projections of optical sections) of LDs stained with the neutral lipid-selective fluorescent dye BODIPY (green) in cotyledon cells near the outer edge of an arabidopsis dry, mature seed embryo (top panel) and an arabidopsis seedling 2 days after the onset of germination (bottom panel). Bar, 5 μm. Top row, right panel: TEM image of cotyledon cells in a germinated (5 days after imbibition) cucumber (*Cucumis sativus*) seedling. Note the LD that is appressed to a peroxisome (arrowheads), presumably to facilitate the transport of fatty acids during lipolysis and provide membrane lipids for peroxisome expansion. Bar, 1 μm. Bottom row, from left to right and as indicated by panel labels: CLSM images (shown as either single or Z-stack projections of optical sections) of BODIPY-stained LDs (green) in a germinated arabidopsis pollen grain and growing pollen tube. Bar, 5 μm. Root epidermal and leaf mesophyll cells (below the adaxial surface of the leaf) of an arabidopsis 15-day-old seedling. Note that the LDs in the (palisade) mesophyll cells are located outside chloroplasts (chlorophyll autofluorescence, false colored blue). Bars, 20 μm. Tobacco (*Nicotiana tabacum*) Bright Yellow-2 suspension-cultured cells incubated with the fatty acid linoleic acid to induce LD proliferation [28]. Bar, 10 μm. (C) CLSM merged image of LD-associated protein 3 (LDAP3)-Cherry (comprising LDAP3 fused at its C terminus to the RFP Cherry [28]) and BODIPY-stained LDs in a leaf guard-cell complex of a 15-day-old stably transformed arabidopsis seedling. The box in the left panel represents the portion of the cells shown at higher magnification in the right panel. Note that the LDAP3-Cherry (red) surrounds the BODIPY-stained TAG core (green), indicating that LDAP3 is localized to the surface of the LDs. Bar, 20 μm. (D,E) LD abundance in arabidopsis leaves during the diurnal cycle and in response to temperature stress. CLSM images (Z-stack projections of optical sections) of BODIPY-stained LDs (green) in mesophyll cells of 15-day-old seedlings at either the end of the day (when the number of LDs is lowest) or the end of the night (when the number of LDs is highest) (D) or before (0 h) or after heat stress (HS) at 37°C for 1 h or cold stress (CS) at 4°C for 24 h (E). Refer to [28] for additional details. Note in (D) the differences in the morphology of chloroplasts (blue) between the end of day and the end of night due to light-induced chloroplast relocalization and (thylakoid) membrane remodeling. Bars in (D,E), 20 μm.

Table 1. Plant LD-Related Proteins Discussed in This Review

Proteins	Refs
α-DOX1	[37]
Caleosin[a]	[37–40]
CGI-58[b]	[55,62,74,111]
LDAP1–3	[25,27,28,32,33]
OBAP1	[41]
Oleosin	[14–22]
PXA1[b]	[62–67,72,73]
SEIPIN 1–3[b]	[49]
HSD1	[36]
SDP1[b]	[68,70–71]

[a]Includes caleosin family members CLO3, CLO4, and responsive to dehydration 20 (RD20).
[b]Proteins involved in LD biogenesis but not localized at steady state to LDs. SEIPINs are ER integral membrane proteins localized at ER–LD junctions and are involved in LD formation; CGI-58, PXA1, and SDP1 are localized to peroxisomes where they participate in LD turnover. See text for additional details.

LD-associated proteins (LDAPs) to connote a more generalized function in neutral lipid compartmentalization for this family of proteins [28]. Like the oleosins the LDAPs are specific to plants and algae and in arabidopsis the LDAPs are ubiquitously expressed and targeted specifically to the surface of LDs in a variety of cell types, including in leaves (Figure 1C) [28]. Furthermore, *LDAP* overexpression and suppression studies resulted in corresponding increases and decreases in the number of LDs, respectively, revealing a role for LDAPs in modulating LD abundance [28]. Characterization of the arabidopsis LDAPs also revealed that LD abundance in leaves is modulated throughout the diurnal cycle (Figure 1D) [28], suggesting that LDs may be coupled to other metabolic processes, including breakdown of transient starch during the night [29] and light-induced stomatal opening [30].

Besides being required for the proper compartmentalization of LDs, LDAPs are also involved in temperature stress responses, characterized by proliferation of LDs in arabidopsis leaves (Figure 1E) and increases in *LDAP* expression [28]. Conversely, *ldap* mutants possessed fewer LDs in leaves following temperature stress [28]. LDAPs also appear to play an important role in drought stress [31,32] and plant growth and development [32,33]. Given their role in modulating neutral lipid compartmentation and LD abundance in plant cells, it is possible that LDAPs and associated LDs are involved in active membrane remodeling and/or lipid-mediated signaling processes.

The identification of other LD proteins is further illuminating new roles for LDs in plants. In seeds several LD proteins besides oleosins are often identified in proteomic analyses, including the caleosins and steroleosins [34,35]; the latter are also referred to as sterol dehydrogenases (HSDs). While the roles of these proteins in seeds are not entirely clear, their presence and characterization in LDs in vegetative cell types is revealing new aspects of LD function. In rice a recently characterized HSD was localized to both the ER and the LD surface and was shown to be involved in cuticular lipid formation and modulation of soluble fatty acid content in leaves [36]. In arabidopsis two enzymes that localize to the LD surface, a caleosin with peroxgenase activity and α-dioxygenase 1 (α-DOX1), catalyze coupled reactions to produce the antifungal compound 2-hydroxyoctadecanoic acid [37,38]. Two other caleosins are also involved in plant stress responses [39,40]. Another LD protein called oil body protein associated 1 (OBAP1) was

recently identified in maize, and disruption of the homolog in arabidopsis resulted in abnormal LD formation in seeds and decreased seed oil content and germination rate [41]; however, while ectopically expressed OBAP1 was shown to target to LDs in leaves [41], its role in LD biogenesis and function in vegetative cell types is unknown.

Taken together, these and other findings have dismissed the notion that LDs are merely inert depots for neutral lipids. Undoubtedly the future identification of additional plant LD proteins, including those in oil-rich vegetative tissues [42] or homologs of proteins identified in LD proteomes of algae [43] or non-plant organisms [44], will continue to reveal other novel aspects of LD function and biogenesis.

Lipodystrophy-Like Genes Modulate LDs and Neutral Lipids in Plants

The cellular machinery governing the biogenesis of LDs appears to be conserved conceptually between kingdoms. This conservation has allowed researchers to identify LD-related proteins in plants based on studies describing proteins from other organisms known to be involved in LD biology and/or lipid metabolism. Analyses of plant homologs of proteins associated with human neutral lipid storage disorders (i.e., lipodystrophy syndromes) have proved to be an especially fruitful strategy.

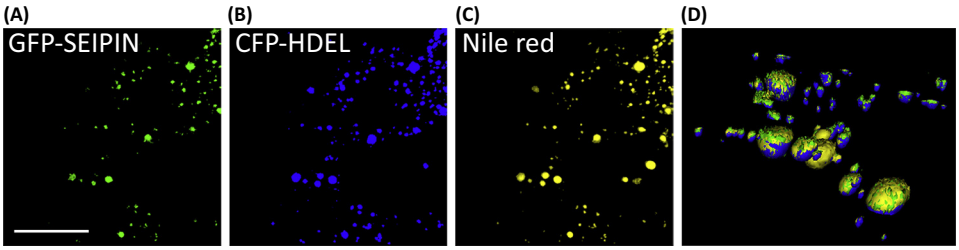
SEIPIN: A Biogenetic Protein Determining LD Number and Size

Mutation of the *SEIPIN* gene in humans, also known as *BCSL2*, leads to a severe form of congenital generalized lipodystrophy characterized by severe insulin resistance, hypertriglyceridemia, and the near absence of adipose tissue [45]. At the cellular level, *SEIPIN*-deficient mammalian and yeast (*Saccharomyces cerevisiae*) cells (*sei1*, formerly *fld1*) produce numerous abnormally small LDs despite retaining the capacity for normal lipid synthesis and turnover [46–48]. Three *SEIPIN* homologs exist in arabidopsis (*AtSEIPIN1–3*) [49] and the products of all three genes could partially recover normal LD formation in the yeast *sei1* mutant [49] and, like mammalian and yeast SEIPINs [48,50–52], localized to ER–LD junctions (Figure 2) [49]. Furthermore, overexpression of each homolog in either yeast cells or plant leaves influenced the number and size of LDs differently. For example, *AtSEIPIN3*-overexpressing cells produced a large number of smaller LDs, whereas cells overexpressing *AtSEIPIN1* produced fewer but larger LDs. Overexpression of *AtSEIPIN1* in arabidopsis also resulted in increases in seed size and seed oil content by as much as 10% [49].

While the precise mechanism by which plant SEIPINs promote LD biogenesis remains to be elucidated, in mammalian and insect cells the protein has been shown to play a key role at ER–LD junction sites by facilitating the incorporation of lipids (and perhaps proteins) during the growth of small, nascent LDs into mature LDs, and hence influence the overall size of mature LDs (Box 1) [48,51,52]. Consistent with this premise, analyses of various N-terminal truncated and swapped versions of the arabidopsis SEIPINs, along with their yeast counterpart, revealed that the small and large LD phenotypes were dictated by each protein's distinct N-terminal domain [49]. Based on these data and those from mutagenic studies of yeast SEIPIN [53,54], it may be that the N terminus of SEIPIN mediates its organization in the ER membrane, specifically at ER–LD junction sites, and in doing so allows SEIPIN to function as a 'vent' for the release of cargo lipids from within the ER bilayer into the emerging LD.

COMPARATIVE GENE IDENTIFICATION-58 (CGI-58): A Key Modulator of Neutral Lipid Content and LD Abundance

Like SEIPIN, the importance of CGI-58 in LD biogenesis in mammals prompted researchers to study its plant homologs [55]. Disruption in humans of *CGI-58*, also known as *α/β-hydrolase domain 5* (ABDH5), results in a rare neutral lipid storage disorder known as Chanarin–Dorfman syndrome [56], which is characterized by ectopic intracellular accumulation of neutral lipids in



Trends in Plant Science

Figure 2. Localization of SEIPIN to Endoplasmic Reticulum (ER)–Lipid Droplet (LD) Junction Sites in Plant Cells. Confocal laser-scanning microscopy (CLSM) images of tobacco (*Nicotiana benthamiana*) leaf mesophyll cells transiently transformed with (A) all three arabidopsis SEIPINs fused to GFP at their N termini and (B) the ER marker protein CFP-HDEL (blue), which comprises CFP linked to the yeast Kar2p (BiP) N-terminal signal sequence and a C-terminal HDEL ER retrieval signal. (**C**) LDs were stained with the neutral lipid dye Nile red and false colored yellow. Images shown in (A–C) are Z-stack projections of optical sections of (palisade) mesophyll cells just below the adaxial surface of the leaf. (**D**) is a 3D projection of surface rendered, high-magnification Z-stack images of a selected region of the same cell showing the localization of the GFP-SEPINs to LDs that are also associated with the ER. Bars in top and bottom panels, 20 μm and 5 μm, respectively. Images reproduced from [49] (www.plantcell.org; ©American Society of Plant Biologists).

organs, tissues, or cells that typically do not store lipids, such as skin and blood cells [57,58]. In mammals CGI-58 is known to be a key regulator of LD turnover via cellular lipases (a process referred to as **lipolysis**) (reviewed in [59]) and its activity is modulated in part through its physical interaction with perilipin (Figure 3A) [60,61]. Although plants lack any obvious homologs of perilipin, disruption of the arabidopsis *CGI-58* homolog yielded a Chanarin–Dorfman syndrome-like phenotype in leaves [55]. That is, while the seed oil content in *cgi-58*-mutant plants was unaffected, the leaves of the plant had an elevated neutral lipid content and substantially increased numbers of LDs [55].

In mammals CGI-58 remains bound to perilipin at the LD surface until adrenergic stimulation promotes phosphorylation of perilipin, which releases CGI-58 and allows it to interact with and stimulate the activity of adipose TAG lipase (ATGL), and the resulting free fatty acids are subsequently broken down in mitochondria (Figure 3A) [59]. In plants fatty acids are degraded in peroxisomes and CGI-58 was discovered to modulate cellular neutral lipid content primarily by interacting with and presumably influencing the activity of peroxisomal ABC transporter 1 (PXA1) rather than mediating TAG turnover by stimulating lipase activity directly (Figure 3B) [62]. PXA1 is a membrane-bound transporter that regulates fatty acid β-oxidation by facilitating the import of fatty acids into the peroxisome [63–65] and its disruption results in steady-state increases in neutral lipid content and LDs in leaves [66,67], with a similar but less severe phenotype observed in *cgi-58*-mutant plants [55]. Up to 90% of the fatty acids made available to PXA1 are supplied by the TAG lipase sugar-dependent 1 (SDP1) [68] (reviewed with other plant TAG lipases in [69]). SDP1 is localized to the peroxisomal surface and during the early stages of post-germinative seedling growth appears to come into contact with LDs via the close interactions that develop between these two organelles [70,71], possibly facilitated by peroxisomal membrane extensions called ‘peroxules’ [71] (Figure 3B).

Thus, while CGI-58 regulates LD turnover and neutral lipid homeostasis similarly in plants and animals, it does so by interacting with different protein partners, reflecting the absence of perilipins in plants and the differences in compartmentalization between fatty acid degradation pathways in peroxisomes and mitochondria. Notably, PXA1 also mediates the uptake of lipophilic precursors for jasmonate and auxin signaling [72,73] and CGI-58 also participates in these lipid-signaling processes [62]. CGI-58 has also been shown to modulate polyamine

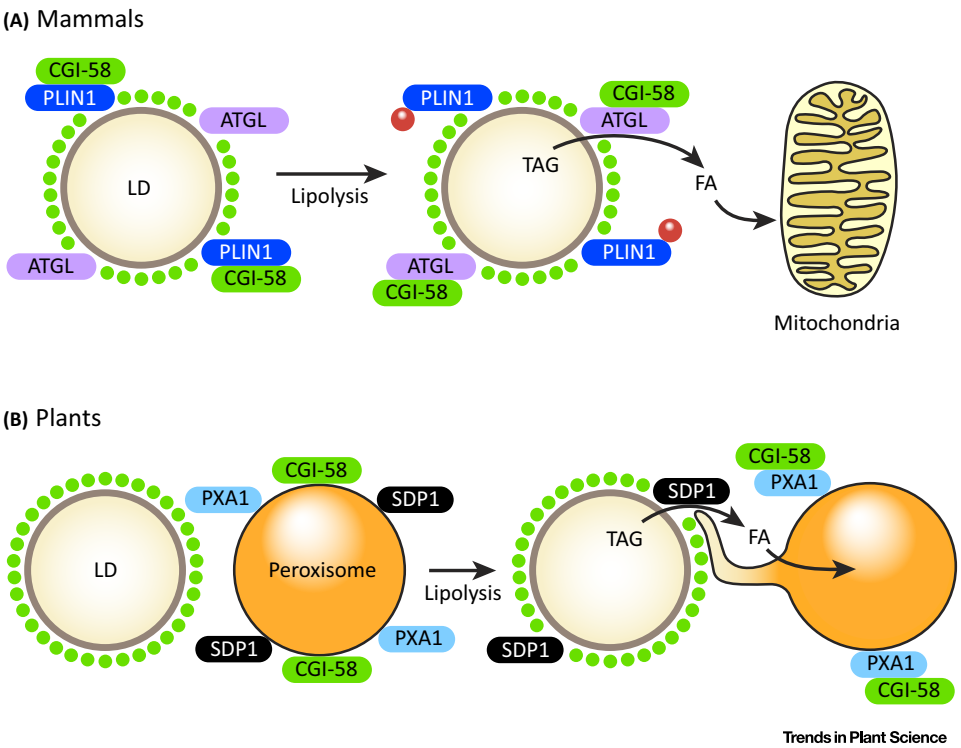


Figure 3. Schematic Representation of Lipolysis in Mammals and Plants. (A) Model of lipolysis in mammalian fat-storing adipocytes. In the absence of a cellular cue for lipolysis, Comparative Gene Identification-58 (CGI-58) (also referred to as ABHD5 in mammals) interacts with perilipin isoform 1 (PLIN1) on the lipid droplet (LD) surface and the activity of adipose triacylglycerol (TAG) lipase (ATGL), which catalyzes the conversion of TAG to diacylglycerol (DAG) and fatty acids (FAs), is low – a process referred to as basal lipolysis. On β-adrenergic stimulation, such as during starvation or exercise, PLIN1 is phosphorylated (red circles), which results in the release of CGI-58 and its subsequent binding to and activation of ATGL, leading to an increase in TAG hydrolysis and β-oxidation of the resulting FAs in mitochondria. While not depicted in the model, lipolysis in mammals also involves several other key protein players, including GO/G1 switch protein 2, which is an attenuator of ATGL during basal lipolysis, hormone-sensitive lipase (HSL) and monoglyceride (MAG) lipase, which hydrolyze DAG and MAG, respectively, to FAs, protein kinase A, which catalyzes the phosphorylation of PLIN1, as well as ATGL and HSL, and FSP27, which binds to ATGL and inhibits lipolysis by decreasing access to ATGL by its coactivator CGI-58. Further, LDs are remodeled during lipolysis into smaller-sized 'micro'-LDs, which increases the LD surface-to-volume ratio and promotes lipase accessibility. See [59] for additional details on lipolysis in mammals. (B) Model of lipolysis in plant cells. Before lipolysis, LDs are protected from cellular lipases by 'coat' proteins, such as the oleosins in seeds. Unlike in mammals, CGI-58 and the major TAG lipase in plants, sugar-dependent 1 (SDP1), are both localized to the peroxisomal surface rather than to LDs. On stimulation of lipolysis, such as in germinated seedlings, LDs and peroxisomes physically interact in a manner that is negatively regulated by sucrose production [70] and SDP1 comes into contact with the LD surface, presumably via peroxisomal membrane extensions (i.e., peroxules) and the retromer complex [71]. FAs resulting from the hydrolysis of TAGs are then transported into peroxisomes via peroxisomal ABC transporter 1 (PXA1) (in association with CGI-58) and metabolized via β-oxidation. Not depicted in the model is the regulated turnover of LD coat proteins during lipolysis, such as the ubiquitin–proteasome-dependent degradation of the oleosins during post-germinative seedling growth [107,108]. See [69] for additional details on lipolysis in plants. Model in (A) based in part on [59].

metabolism in both plants and mammals, revealing additional roles in the modulation of other metabolic activities [74,75].

LD Proteins Unique to Yeast and/or Animals Can Modulate LDs and Neutral Lipids in Plants

Mounting evidence indicates that at least some LD proteins without known homologs in other species may still share functional features in the conserved cellular machinery of LD biogenesis across kingdoms and thus may be used as effective tools to manipulate LD accumulation and

to study the cellular mechanism of LD biogenesis. For example, ectopic expression of several LD proteins, such as the plant oleosins and SEIPINs as well as the human perilipins, was shown to modulate LD morphology and accumulation in yeast (*S. cerevisiae*) [49,76–80]. Likewise, two proteins from *Drosophila*, Brummer and perilipin 1, were recently shown to target and aggregate LDs in tobacco pollen tubes [81]. In the same study, however, two LD proteins from *S. cerevisiae*, ScGtt1p and ScTgl3p, did not localize to pollen tube LDs [81], indicating that the molecular mechanism underlying LD protein targeting is not entirely conserved for all LD proteins.

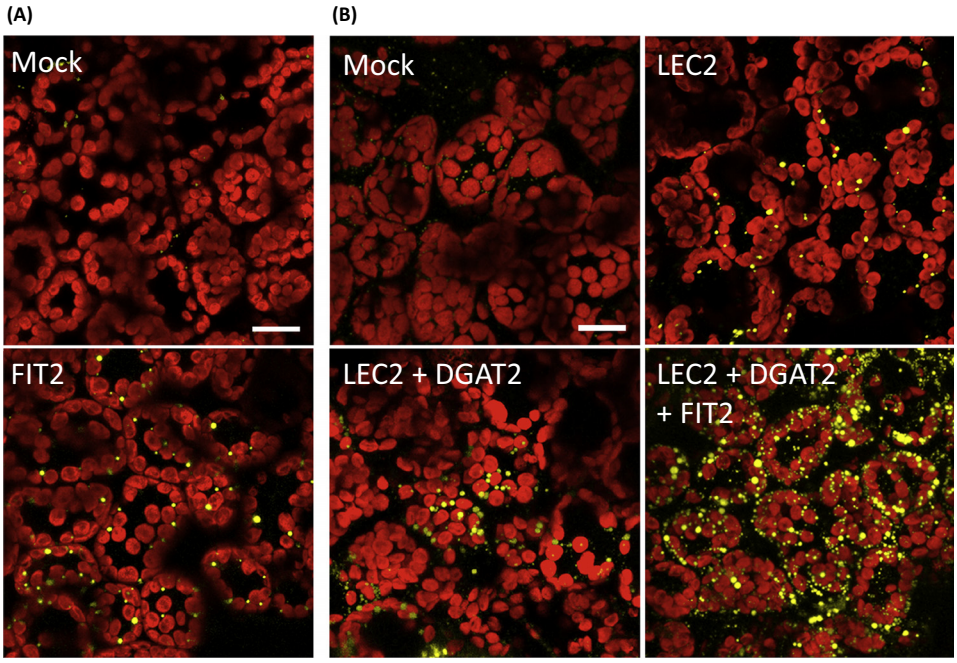
Evidence also exists that LD proteins unique to yeast and/or animals may be able to interact with the endogenous LD biogenesis machinery in plants to influence LD formation. Fat storage-inducing transmembrane protein 2 (FIT2) in animal cells is an integral ER membrane protein that plays an important role in LD biogenesis by binding to and partitioning TAGs into growing LDs (Box 1) [82–84] and while homologs of FIT2 have been identified in a wide range of organisms, including yeast, *Caenorhabditis elegans*, zebrafish, mouse, and human [82], no apparent homologs of FIT2 exist in plants [1]. Nonetheless, mouse FIT2 was able to specifically target to the ER, possibly to ER–LD junction sites, and promote LD accumulation when expressed in plant cells (Figure 4A) [85]. Transiently-expressed mouse FIT2 also colocalized with diacylglycerol (DAG) acyltransferases (DGATs) in specific regions (subdomains) of the ER in plant cells and stable ectopic expression of mouse FIT2 in arabidopsis plants increased oil content in the leaves and seeds of several transgenic lines [85]. Hence, it appears that FIT2 functions within plant systems to promote TAG accumulation and storage in LDs, and it may be possible to use FIT2 to identify cross-kingdom protein factors of LD biogenesis.

Besides FIT2 there are several other LD proteins that are unique to yeast and/or animals and that may function when produced in plants [1]. One example is fat-specific protein 27 (FSP27) [also referred to as cell death-inducing DNA fragmentation factor α-like effector c (CIDEC)], which functions in mammals to regulate the size of LDs by mediating LD fusion (Box 1) [86] as well as serving as a negative regulator of lipolysis [87]. Organism-specific LD proteins like FSP27 or FIT2 might be introduced into plant cells to probe the cellular nature and extent of overlap with the LD biogenetic machinery of plants. As discussed in more detail below, the heterologous expression of at least one of these unique LD proteins in plants (i.e., FIT2) also provides an intriguing strategy to enhance the packaging of neutral lipids in plants.

Engineering LDs for Production of Biofuels and Bioproducts

As our understanding of LD biogenesis increases, there are numerous opportunities to consider how this information might be applied to engineering the production of value-added lipids and proteins in plants. Knowledge of oleosins as LD coat proteins has already led to strategies for the large-scale purification of desired proteins by exploiting the specific targeting of oleosin fusion proteins to LDs and the buoyant density of the LD particle [88].

There is also a potential to elevate the oil content of plants to help meet the need for plant oils as a feedstock for **biofuels and bioproducts** [89]. Significant portions of some oilseed crops are already used for biodiesel production but demand for fuel is far greater than what agriculture can typically deliver, which puts significant pressure on food and feed production systems. A promising alternative strategy is to elevate oil content in the vegetative organs of dedicated non-food bioenergy crops. Neutral lipids typically account for less than 0.5% of leaf dry weight, but bioengineering strategies have been developed to 'push' more carbon into fatty acid biosynthetic pathways in leaves, 'pull' more fatty acids into neutral lipid TAG synthesis, and 'protect' TAGs from turnover, such as by disruption of *CGI-58* and *PXA1* [90–92]. Overall these studies have revealed that plants are remarkably amenable to accumulating greater amounts of oil in leaves, with up to 15–30% dry weight in leaves reported for engineered tobacco [93,94]. Similar



Trends in Plant Science

Figure 4. Expression of Mammalian Fat Storage-Inducing Transmembrane Protein 2 (FIT2) Promotes Proliferation of Lipid Droplets (LDs) in Plant Cells, Which Is Further Enhanced by Coexpression with Other Lipid-Inducing Proteins. (A) Confocal laser-scanning microscopy (CLSM) images of tobacco (*Nicotiana benthamiana*) leaf mesophyll cells transiently transformed either without (referred to as 'mock' transformation) or with mouse (*Mus musculus*) FIT2. LDs were stained with Nile red (false colored yellow) and the autofluorescence attributable to chloroplasts (chlorophyll) is colored red. Note the relative increase in LDs in the cells expressing FIT2. (B) 'Push–pull–package' strategy to increase the abundance of LDs in leaves. CLSM images of tobacco leaf mesophyll cells either mock transformed or transiently (co)transformed with arabidopsis LEAFY COTYLEDON 2 (LEC2) alone, which induces genes for seed oil-like synthesis in leaves (i.e., 'push') [109], with LEC2 and mouse diacylglycerol acyltransferase 2 (DGAT2), the latter of which catalyzes the synthesis of triacylglycerol (TAG) and induces an increase in oil content in leaves (i.e., 'pull') [93,98,110], or with LEC2, DGAT2, and FIT2, which promotes the compartmentalization of neutral lipids (i.e., TAG) into LDs at the endoplasmic reticulum (ER) and induces an increase in oil content (and LDs) in leaves (i.e., 'package') [85]; refer also to (A). All transformations in (A,B) included the viral suppressor of transgene silencing p19 and all images are Z-stack projections of optical sections of palisade mesophyll cells just below the adaxial surface of the leaf. Bars, 20 μm.

'push, pull, and protect' strategies were used to increase oil content up to 3.3% dry weight in potato tubers [95,96].

Recent studies of LD biogenesis have further revealed a somewhat unexpected result that the promotion of LD formation results in an increase in the steady-state amount of leaf oil. That is, it appears that if neutral lipid storage 'compartments' are formed, cells respond by filling them. This shifts the equilibrium towards the accumulation of more neutral lipid. SEIPIN promotes the formation of LDs and while the protein is not involved in neutral lipid synthesis directly, its overexpression in plants results in an increase in LDs and an approximate doubling of the amount of neutral lipid [49]. Similar increases are observed with overexpression of oleosins [97], LDAPs [28], or mouse FIT2 [85] in plant cells. An obvious next step would be to include genes such as *SEIPIN*, *LDAP*, and *FIT2* in push, pull, and protect strategies to add a 'package' component to perhaps further elevate the capacity of plant leaves to store oil. An example is provided in Figure 4B, whereby the LEAFY COTYLEDON 2 (LEC2) transcription factor pushes more carbon into fatty acid biosynthetic pathways, DGAT2 pulls more of the cellular fatty acids towards TAG synthesis, and FIT2 promotes the packaging of TAG into LDs in tobacco leaves.

While the majority of experiments to date have focused on increasing the total amount of oil in plant leaves, there are also opportunities to consider modulating the composition of the oil found within the LD. Recently, Yurchenko *et al.* [98] reported on the coupled expression of an enzyme for the synthesis of a 'conjugated' fatty acid (i.e., eleostearic acid) that is typically not present in plant leaves but has important nutritional and industrial applications, with DGAT2, which can 'pull' this unusual fatty acid into neutral lipid biosynthetic pathways. This led to synergistic increases in total leaf oil content, number of LDs, and eleostearic acid accumulation. The ability to manipulate the fatty acid composition of neutral lipids in leaves opens the door to the production of many different types of oils in plants, which expands the diversity and value of the oils beyond just biofuels [99,100].

Concluding Remarks and Future Perspectives

Recent studies have greatly expanded our view of the LD beyond simply an inert storage depot for neutral lipids. Cytoplasmic LDs are dynamic organelles present in essentially all cell types and are involved in diverse biological processes including stress responses, diurnal and light-regulated processes, and plant growth and development. These discoveries have been driven in part by recent proteomics [25] and homology-based [49,55] studies of LDs in non-seed tissues, revealing new proteins involved in LD biogenesis and function in plants. The roles of LDs in these various processes is only beginning to emerge, raising important questions about LD biology in general (see Outstanding Questions). Furthermore, analysis of these new proteins, including their use as probes to identify other interacting protein partners, such as CGI-58's interaction with PXA1 [62], will expand our knowledge of their underlying molecular mechanisms. There are also other, lipodystrophy-related proteins in plants that have not yet been characterized [1] and additional proteomics studies, especially those aimed at non-seed tissues, will undoubtedly continue to reveal new LD protein constituents.

The identification of new protein machinery also provides molecular tools to engineer increases in the neutral lipid content and energy density of crops. The ability of unique non-plant proteins, like mammalian FIT2, to induce LD formation in plants (Figure 4) [85] suggests that cross-kingdom proteins can be combined in synthetic biology strategies to enhance oil content and composition. Given the emerging additional physiological roles of LDs, it is likely that we will see a convergence of engineering strategies that not only increase oil content in plants but also increase tolerance to biotic and/or abiotic stress and thereby develop crops that serve as more sustainable sources of food, fuel, and other value-added lipophilic chemicals.

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Outstanding Questions

Are LDs in plants, similar to their counterparts in mammals and insects, formed at the ER in a stepwise manner that involves the formation of nascent LDs and their conversion to mature LDs? Do mature LDs in plants always dissociate into the cytoplasm or can they also remain permanently attached to the ER?

Are there specific regions (subdomains) of the ER where LD formation occurs?

Do plant cells have a protein functionally equivalent to FIT2?

Are different classes of neutral lipids packaged together into LDs or do they remain separated in cells by their contents?

How do LDs detach from the ER, if at all? Given that detachment of LDs is conceptually the same as that of transport vesicles and that, at least in mammals, the Arf1–COPI machinery participates in several ways in LD biogenesis (e.g., development of LD–ER junctions, LD protein targeting, lipolysis), could Arf1–COPI also be involved in LD formation?

Do plant LDs, similar to their mammalian and insect counterparts, undergo an expansion phase whereby enzymes involved in lipid biosynthesis traffic from the ER directly to the LD surface to stimulate LD growth?

What is the nature of the molecular targeting information and machinery that allows LD proteins to specifically localize to LDs?

What are the roles of LDs during the diurnal cycle and how does their relative abundance relate to general carbon metabolism?

What are the roles of LDs during abiotic stress responses? Do LDs that proliferate in response to temperature participate in lipid signaling by providing (and/or housing) lipid metabolites or perhaps by serving as a surface for the scaffolding of proteins involved in signal transduction? Do LDs provide fatty acids or glycerolipids for the membrane remodeling required to help maintain the physical and functional aspects of membranes during

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stress? Do LDs help buffer the cytotoxicity of the free fatty acids often generated during plant stress by serving as reservoirs for newly synthesized TAG?

What are the roles of LDs during biotic stress responses? In addition to the questions raised above for abiotic stress responses, do LDs help traffic oxylipins from their site of synthesis in the chloroplast to the peroxisome for their subsequent conversion into jasmonic acid and/or other downstream lipid-signaling molecules?

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